regulation wherein binding of Src-family kinase SH3 domains to p85 increased enzymatic activity and indicate that this mechanism is operative during antigen receptor signaling.

Cross-linking of CD19 and membrane IgM (mIgM) on B cells induces tyrosine phosphorylation of Tyr-X-X-Met motifs on CD19 and association of CD19 with PI-3 kinase (*27*). Thus, PI-3 kinase activation through Src-family kinase SH3 domains may be amplified by the binding of tyrosine-phosphorylated CD19 to p85 SH2 domains. Each of these mechanisms could localize PI-3 kinase to the membrane, which may be important for enzyme access to substrate (*31*).

REFERENCES AND NOTES

1. J. C. Cambier and J. T. Ransom, *Annu. Rev. Immunol.* **5**, 175 (1987); A. L. DeFranco, *Annu. Rev. Cell Biol.* **3**, 143 (1987); *Nature* **342**, 340 (1989); C. C. Goodnow, J. Crosbie, H. Jorgensen, R. A. Brink, A. Basten, *ibid.*, p. 385; D. A. Nemazee and K. Buerki, *ibid.* **337**, 562 (1989).
2. A. L. Burkhardt, M. Brunswick, J. B. Bolen, J. J. Mond, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7410 (1991); Y. Yamanashi, T. Kakiuchi, J. Mizuguchi, T. Yamamoto, K. Toyoshima, *Science* **251**, 192 (1991).
3. Y. Yamanashi, Y. Fukui, B. Wongsasant, Y. Kinoshita, Y. Ichimori, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1118 (1992).
4. J. A. Escobedo *et al.*, *Cell* **65**, 75 (1991); M. Otsu *et al.*, *ibid.*, p. 91; E. Y. Skolnik *et al.*, *ibid.*, p. 83.
5. I. D. Hiles *et al.*, *ibid.* **70**, 419 (1992).
6. M. Whitman, C. P. Downes, M. Keeler, T. Keller, L. Cantley, *Nature* **332**, 644 (1988).
7. P. J. Parker and M. D. Waterfield, *Cell Growth Differ.* **3**, 747 (1992).
8. M. Whitman, D. R. Kaplan, B. Schaffhausen, L. Cantley, T. Roberts, *Nature* **315**, 239 (1985).
9. M. Whitman, D. R. Kaplan, T. M. Roberts, L. Cantley, *Biochem. J.* **247**, 165 (1987).
10. G. Endemann, K. Yonezawa, R. A. Roth, *J. Biol. Chem.* **265**, 396 (1990).
11. L. Varticovski, B. Druker, D. Morrison, L. Cantley, T. Roberts, *Nature* **342**, 699 (1989).
12. S. R. Coughlin, J. A. Escobedo, L. T. Williams, *Science* **243**, 1191 (1989); J. Fantl *et al.*, *Cell* **69**, 413 (1992); A. Kashishian, A. Kazlauskas, J. A. Cooper, *EMBO J.* **11**, 1373 (1992); A. Kazlauskas and J. A. Cooper, *Cell* **58**, 1121 (1989); A. Kazlauskas, A. Kashishian, J. A. Cooper, M. Valius, *Mol. Cell. Biol.* **12**, 2534 (1992); S. Lev, D. Givol, Y. Yarden, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 678 (1992); M. Reedijk, X. Liu, T. Pawson, *Mol. Cell. Biol.* **10**, 5601 (1990); M. Reedijk *et al.*, *EMBO J.* **11**, 1365 (1992); S. A. Shurtleff *et al.*, *ibid.* **8**, 2415 (1990); D. A. Talmage *et al.*, *Cell* **59**, 55 (1989).
13. K. R. Auger *et al.*, *Cell* **57**, 167 (1989); H. Hayashi *et al.*, *J. Biol. Chem.* **267**, 22575 (1992); P. Hu *et al.*, *Mol. Cell. Biol.* **12**, 981 (1992); D. R. Kaplan *et al.*, *Cell* **50**, 1021 (1987); S. R. Coughlin, J. A. Escobedo, L. T. Williams, *Science* **243**, 1191 (1989).
14. J. M. Backer *et al.*, *EMBO J.* **11**, 3469 (1992); Y. R. Hadari *et al.*, *J. Biol. Chem.* **267**, 17483 (1992); M. G. Myers *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10350 (1992).
15. M. Valius and A. Kazlauskas, *Cell* **73**, 321 (1993).
16. Y. Fukui and H. Hanafusa, *Mol. Cell. Biol.* **9**, 1651 (1989); J. S. Gutkind, P. M. Lacal, K. C. Robbins, *ibid.* **10**, 3806 (1990); M. C. O'Brien, Y. Fukui, H. Hanafusa, *ibid.*, p. 2855.
17. D. S. Wages, J. Keefer, T. B. Rall, M. Weber, *J. Virol.* **66**, 1866 (1992).
18. H. Hirai and H. E. Varmus, *Cell* **87**, 8592 (1990); C. Seidel-Dugan, B. E. Meyer, S. M. Thomas, J. S. Brugge, *Mol. Cell. Biol.* **12**, 1835 (1992).
19. S. A. Courtneidge and A. Heber, *Cell* **50**, 1031 (1987).
20. C. M. Pleiman *et al.*, *Mol. Cell. Biol.* **13**, 5877 (1993).
21. X. Liu, L. M. Maregere, C. A. Koch, T. Pawson, *ibid.*, p. 5225; K. V. S. Prasad *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7366 (1993).
22. P. Cicchetti, B. J. Mayer, G. Thiel, D. Baltimore, *Science* **257**, 803 (1992); R. Ren, B. J. Mayer, P. Cicchetti, D. Baltimore, *ibid.* **259**, 1157 (1993).
23. H. Yu *et al.*, *ibid.* **258**, 1665 (1992).
24. Peptides to amino acid residues 80 to 104 (KKISPPTPKPRPPRPTPVAPGSSKT) and 299 to 318 (NERQPAPATPPKPPKPTTVA) of the human PI-3 kinase p85α subunit were synthesized on an Applied BioSystems model 430A. Peptide sequence and purity was verified by amino acid hydrolysis analysis (Waters Associates Pico-Tag system). Abbreviations for the amino acid residues are A, Ala; E, Glu; G, Gly; I, Ile; K, Lys; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.
25. C. M. Pleiman and J. C. Cambier, unpublished observations. The antibody to phosphotyrosine (AB2) was obtained from Oncogene Science.
26. M. R. Gold, V. W.-F. Chan, C. W. Turck, A. L. DeFranco, *J. Immunol.* **148**, 2012 (1992).
27. D. A. Tuveson, R. H. Carter, S. P. Soltoff, D. T. Fearon, *Science* **260**, 986 (1993).
28. K. S. Campbell, E. J. Hager, R. J. Friedrich, J. C. Cambier, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3982 (1991).
29. S. G. Ward *et al.*, *J. Biol. Chem.* **217**, 23862 (1992).
30. L. C. Cantley *et al.*, *Cell* **64**, 75 (1991).
31. L. Varticovski, G. Q. Daley, P. Jackson, D. Baltimore, L. C. Cantley, *Mol. Cell. Biol.* **11**, 1107 (1991).

32. Supported by USPHS grants AI21768, AI20519, and AI29903.

21 October 1993; accepted 14 January 1994

Requirement for Transcription Factor IRF-1 in NO Synthase Induction in Macrophages

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Production of nitric oxide (NO) by macrophages is important for the killing of intracellular infectious agents. Interferon (IFN)-γ and lipopolysaccharide stimulate NO production by transcriptionally up-regulating the inducible NO synthase (iNOS). Macrophages from mice with a targeted disruption of the IFN regulatory factor–1 (IRF-1) gene (IRF-1$^{-/-}$ mice) produced little or no NO and synthesized barely detectable iNOS messenger RNA in response to stimulation. Two adjacent IRF-1 response elements were identified in the iNOS promoter. Infection with *Mycobacterium bovis* (BCG) was more severe in IRF-1$^{-/-}$ mice than in wild-type mice. Thus, IRF-1 is essential for iNOS activation in murine macrophages.

Nitric oxide is an unstable free radical gas that functions as an intercellular messenger in vasodilation and neurotransmission and as a cytotoxic agent of macrophages (*1*). Three related genes encode NO synthases (NOS) in different tissues. Endothelial and neuronal NOS are constitutive enzymes whose functions are activated by Ca^{2+}-calmodulin. Macrophages and some other cells have a transcriptionally inducible form of NOS (iNOS) that remains undetectable until these cells are activated. Interferon-γ and bacterial lipopolysaccharide (LPS) are the most potent activators of the iNOS gene in murine macrophages (*2*). Induced NO production is one of the principal mechanisms of macrophage cytotoxity for tumor cells, bacteria, protozoa, helminths, and fungi (*3*); NO also participates in antiviral defenses (*4*). The promoter region of the murine iNOS gene is responsive to stimulation by IFN-γ and LPS on transfection into a murine macrophage cell line (*5*, *6*).

Interferon regulatory factor–1 (IRF-1) and the related IRF-2 protein bind to sites within the promoters of IFN-α, IFN-β, and several IFN-inducible genes (*7*–*12*). IRF-1 activates transcription whereas IRF-2 inhibits it (*8*, *9*). The IRF-1 gene is induced by treatment with IFNs, and also by tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-6, and leukemia inhibitory factor (*13*). Mice were generated with homozygous targeted disruptions of the IRF-1 or IRF-2 genes (*14*). IRF-1$^{-/-}$ mice show no overt abnormalities either during embryonic development or postnatally. The major defect found in IRF-1$^{-/-}$ mice was a 90% reduction in the number of CD8$^+$ T cells. Although embryonic fibroblasts from IRF-

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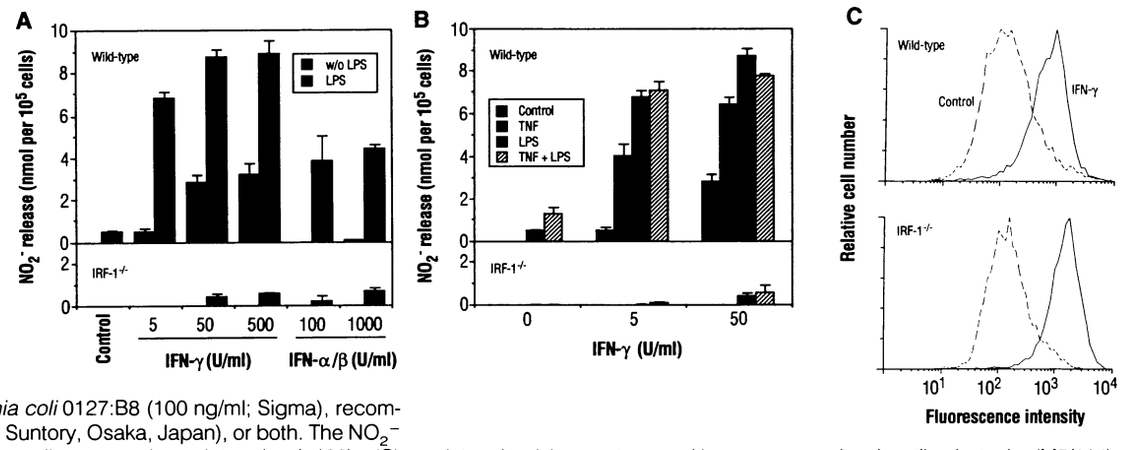

Fig. 1. Induction of NO_2^- release and MHC class II antigen expression in peritoneal macrophages from wild-type and IRF-$1^{-/-}$ mice. (**A** and **B**) Thioglycollate-elicited adherent peritoneal macrophages were cultured for 48 hours in the presence or absence of recombinant murine IFN-γ (Genzyme, Cambridge, Massachusetts) or natural murine IFN-α/β (Lee Biomolecular, San Diego, California), with or without LPS from *Escherichia coli* 0127:B8 (100 ng/ml; Sigma), recombinant murine TNF (100 ng/ml; Suntory, Osaka, Japan), or both. The NO_2^- concentration in the culture medium was then determined (*30*). (**C**) Thioglycollate-elicited, adherent peritoneal macrophage cultures were incubated in the presence or absence of recombinant murine IFN-γ (100 U/ml) for 48 hours, and the expression of MHC class II antigen was determined by treatment with a rat monoclonal antibody to Ia (M5/114), staining with fluorescein isothiocyanate–conjugated goat antibody to rat immunoglobulin G (Boehringer Mannheim, Indianapolis, Indiana), and flow cytometric analysis on a FACScan apparatus (Becton Dickinson) (*16*).

IRF-$1^{-/-}$ mice produced less type I IFN in response to double-stranded RNA, the induction by IFN-α of several genes (the 2′,5′ oligoadenylate synthetase, double-stranded RNA-dependent protein kinase, 1-8 and H-2K^b genes) in fibroblasts from IRF-$1^{-/-}$ mice was not altered, suggesting that IFN-stimulated gene factor–3 (ISGF-3) (*15*) or other IFN-activated transcription factors are sufficient to mediate induction of these genes. In the present study we have used IRF-$1^{-/-}$ mice to analyze the role of IRF-1 in NO generation and induced expression of the iNOS gene in murine macrophages.

Production of NO_2^- (the stable end product of NO synthesis) was examined in peritoneal macrophages from wild-type and IRF-$1^{-/-}$ mice (Fig. 1A). A concentration-dependent induction of NO_2^- by IFN-γ, augmented by the addition of LPS, was seen in macrophages from wild-type mice, as described (*2*, *16*). In contrast, macrophages from IRF-$1^{-/-}$ mice produced no detectable NO_2^- in response to IFN-γ or LPS alone and generated barely detectable NO_2^- after simultaneous treatment with high doses of IFN-γ and LPS. IFN-α/β, though less potent than IFN-γ, induced NO_2^- release in the presence of LPS from wild-type macrophages, as reported (*2*, *16*). This stimulatory effect of IFN-α/β was decreased in macrophages from IRF-$1^{-/-}$ mice. Treatment with TNF augmented IFN-γ–induced NO_2^- production in wild-type macrophages, but not in macrophages from IRF-$1^{-/-}$ mice (Fig. 1B). No reduction in the IFN-γ-induced expression of major histocompatibility (MHC) class II antigen was seen in IRF-$1^{-/-}$ macrophages (Fig. 1C), indicating that these cells are not globally unresponsive to IFN.

Increased NO production in activated murine macrophages is due to increased synthesis of the iNOS enzyme (*1*, *17*). Therefore, we compared iNOS mRNA in macrophages from wild-type and IRF-$1^{-/-}$ mice at different times after stimulation with IFN-γ and LPS (Fig. 2). The increase in iNOS mRNA, seen

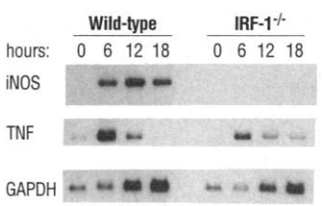

Fig. 2. Induction of iNOS mRNA in peritoneal macrophages from wild-type and IRF-$1^{-/-}$ mice treated with IFN-γ and LPS. Thioglycollate-elicited, adherent peritoneal macrophages (*30*) were incubated with recombinant murine IFN-γ (100 U/ml) and LPS (100 ng/ml) for 6, 12, and 18 hours. Total RNA was isolated by acid guanidium thiocyanate–phenol-chloroform extraction (*31*), separated by electrophoresis (15 μg per sample) through a 1% agarose gel, and blotted onto a nylon membrane. The blot was hybridized to ^{32}P-labeled cDNA probes for iNOS (*17*) and GAPDH. The membrane was exposed to x-ray film for 7 days. Thereafter, the membrane was stripped, and the blot was reprobed with a murine TNF-α probe (*32*) and exposed to x-ray film for 2 days.

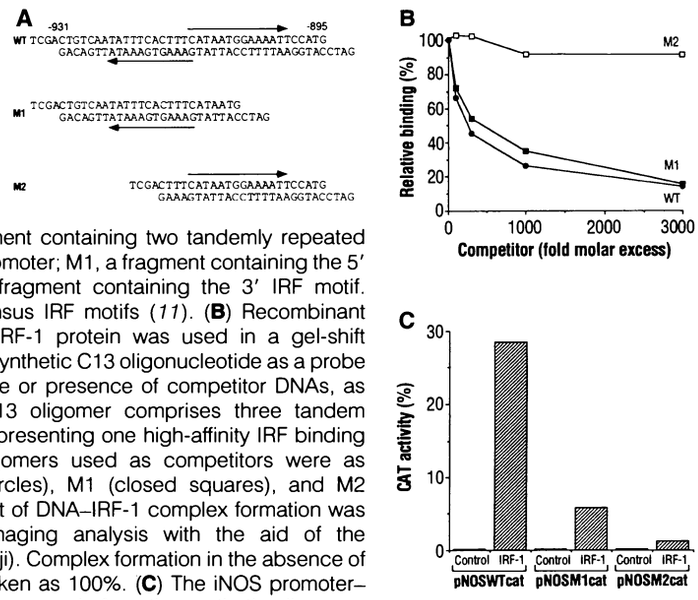

Fig. 3. Identification of IRF-1 sites in the iNOS promoter. (**A**) Sequences of the synthetic oligomers containing IRF motifs from the mouse iNOS promoter. The synthetic oligomers are as follows: WT, a fragment containing two tandemly repeated IRF sites of the iNOS promoter; M1, a fragment containing the 5′ IRF motif; and M2, a fragment containing the 3′ IRF motif. Arrows indicate consensus IRF motifs (*11*). (**B**) Recombinant baculovirus-produced IRF-1 protein was used in a gel-shift assay with ^{32}P-labeled synthetic C13 oligonucleotide as a probe (*11*, *20*), in the absence or presence of competitor DNAs, as described (*9*). The C13 oligomer comprises three tandem repeats of AAGTGA, representing one high-affinity IRF binding site (*11*, *20*). The oligomers used as competitors were as follows: WT (closed circles), M1 (closed squares), and M2 (open squares). Amount of DNA–IRF-1 complex formation was quantitated by radioimaging analysis with the aid of the BAS2000 apparatus (Fuji). Complex formation in the absence of competitor DNA was taken as 100%. (**C**) The iNOS promoter–CAT fusion constructs were made from the synthetic oligomers WT, M1, and M2, respectively, by ligating a Bam HI–Hind III fragment (−55 to +19 of the human IFN-β promoter) from p-55C1 (*20*), a Sal I–Hind III backbone fragment from pBScat, and one of the Sal I–Bam HI synthetic oligomers whose sequences are shown in Fig. 3A. P19 cultures were transfected with 7.5 μg of the CAT reporter gene and 2.5 μg of the IRF-1 expression vector. CAT assays were done as described (*9*). Results shown are the means of duplicate assays. Similar results were obtained in two other experiments.

in macrophages from wild-type mice at 6, 12, and 18 hours after stimulation, was virtually absent in IRF-$1^{-/-}$ macrophages. Treatment of macrophages with IFN-γ and LPS also leads to the induction of the TNF-α gene (*18*). Induction of TNF-α mRNA, which peaked at 6 hours after stimulation, was seen in macrophages from both wild-type and IRF-$1^{-/-}$ mice, indicating that IRF-$1^{-/-}$ macrophages were responsive to treatment with IFN-γ and LPS. Macrophage activation by IFN-γ and LPS also resulted in some gradual increase in glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in both wild-type and IRF-$1^{-/-}$ macrophages, as described (*19*).

Within the mouse iNOS promoter sequence (*5, 6*), we found two adjacent potential IRF binding motifs (*11*), arranged in opposite orientations (Fig. 3A). Oligomers containing this region were synthesized to determine whether these potential IRF binding motifs were functional. To examine IRF binding to these sequences, we used recombinant IRF-1 protein in a competition gel shift assay with a labeled DNA probe that binds IRF-1 with high affinity (*11, 20*). Formation of the labeled DNA–IRF-1 complex was reduced with increasing concentrations of the oligomer containing the two IRF sites of the iNOS promoter (WT). The 5′ IRF motif alone (M1) was a strong competitor, whereas the 3′ IRF motif alone (M2) showed little, if any, ability to compete.

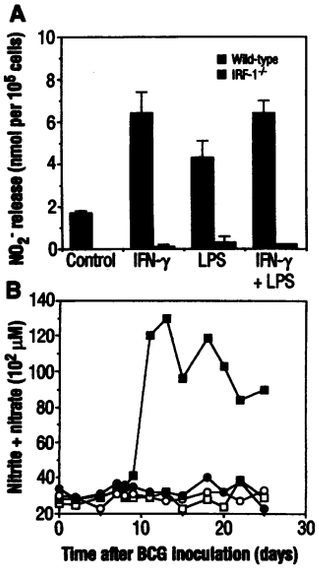

Fig. 4. NO_2^- release from peritoneal macrophages and urinary NO_2^--NO_3^- excretion after BCG infection of wild-type and IRF-$1^{-/-}$ mice. (**A**) Resident peritoneal cells, harvested from mice 2 weeks after intraperitoneal inoculation with BCG (Connaught Laboratories, Swiftwater, Pennsylvania; 1 × 10^6 bacteria per mouse), were isolated by adhesion to plastic dishes and cultured in the absence or presence of IFN-γ (50 U/ml) or LPS (100 ng/ml) or both. NO_2^- release into the medium was determined after 48 hours as in the experiments shown in Fig. 1, A and B. (**B**) Samples of urine were collected from BCG-infected or uninfected wild-type and IRF-$1^{-/-}$ mice (four mice per group) at different times after intravenous inoculation with BCG (2 × 10^7 bacteria per mouse). Urine samples from each group collected at the individual time points were pooled and assayed for NO_2^- content after reduction of NO_3^- to NO_2^- with bacterial nitrate reductase (*21, 22*). Open squares: wild-type, uninfected; closed squares: wild-type, BCG-infected; open circles: IRF-$1^{-/-}$, uninfected; closed circles: IRF-$1^{-/-}$, BCG-infected.

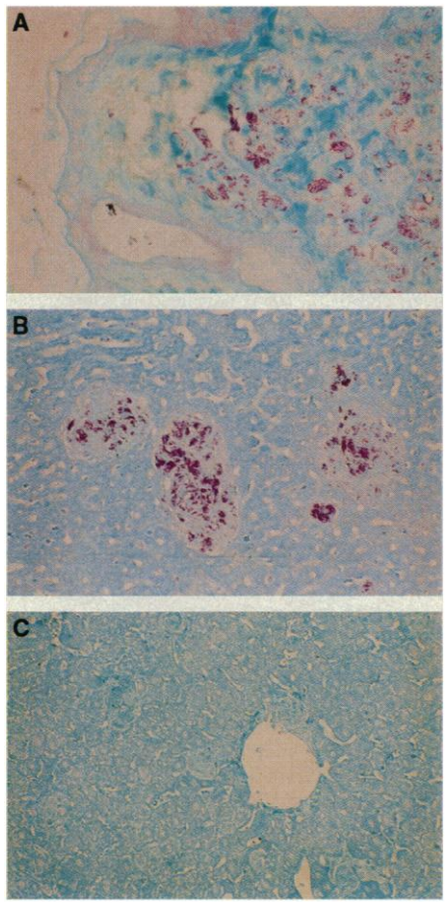

Fig. 5. BCG infection is more extensive in IRF-$1^{-/-}$ mice than in wild-type mice. Results shown are from mice at 9 weeks after intravenous inoculation with BCG (2 × 10^7 bacteria per mouse). Tissues were fixed in 1% formalin and processed to paraffin. Five-micrometer-thick sections were stained with Ziehl-Neelsen's acid-fast stain and counterstained with light green. (**A**) Abundant, characteristically rod-shaped, acid-fast bacteria in skin granulomas of an IRF-$1^{-/-}$ mouse, most of which are localized within epitheloid cells and macrophages (magnification, ×360). (**B**) Acid-fast bacteria in the liver of an IRF-$1^{-/-}$ mouse; many acid-fast bacteria are present, mostly within epitheloid cells and macrophages in the many granulomas in this liver (×180). (**C**) Absence of acid-fast bacteria in the liver of a wild-type mouse. No acid-fast bacteria are present even within the granulomas (×180).

We also constructed reporter genes that comprise the bacterial chloramphenicol acetyltransferase (CAT) gene driven by a promoter and the above-described synthetic oligomers. P19 embryonal carcinoma cells, which are devoid of detectable IRF activity (*9*), were cotransfected with these reporter genes and the IRF-1 expression plasmid, pAct-1 (*9*). The pNOSM2cat construct, which contains the 3′ IRF motif that showed a low affinity for the IRF-1 protein in the gel shift assay, was only weakly activated (Fig. 3C). The pNOSM1cat construct, containing the 5′ IRF motif, showed moderate activation. However, pNOSWTcat, which contains both potential IRF binding sites, showed a high activation. These results suggest that the two IRF binding motifs mediate a synergistic transcriptional activation by IRF-1. Additional studies are needed to show that these two sites are indeed important in the transcriptional activation of the intact iNOS promoter. A region spanning positions −1029 to −913 in the native iNOS promoter (comprising the IRF-1 binding site identified by us) was recently shown to be essential for activation by IFN-γ (*6*).

To determine whether IRF-1 is also important for NO generation in the intact organism, we inoculated wild-type and IRF-$1^{-/-}$ mice intraperitoneally with the BCG strain of *Mycobacterium bovis*. Two weeks later, resident peritoneal macrophages were collected and cultured. In wild-type mice, BCG infection resulted in the mobilization of macrophages that released NO_2^- spontaneously and produced high NO_2^- concentrations in response to IFN-γ or LPS (Fig. 4A). In contrast, macrophages from BCG-infected IRF-$1^{-/-}$ mice produced barely detectable NO_2^-. Mice infected with BCG excrete high concentrations of NO_2^- and NO_3^- in the urine, which peak when activated macrophages appear in the peritoneal cavity (*21, 22*). We found an increase in urinary NO_2^- and NO_3^- concentration 11 to 25 days after BCG infection in wild-type mice, but not in IRF-$1^{-/-}$ mice (Fig. 4B).

Although various mouse strains differ in their degree of resistance to BCG, inoculation with BCG leads to a nonlethal, self-limiting infection even in genetically susceptible mice (*23*). Both TNF-α and IFN-γ are essential for the recovery of mice from BCG infection (*24*). A postulated role for induced NO in host resistance to intracellular infectious agents (*3*), including *M. tuberculosis* (*25*), prompted us to compare the courses of BCG infection in wild-type and IRF-$1^{-/-}$ mice. Three mice from each group were killed 65 to 67 days after intravenous inoculation with BCG. At this time one of the BCG-infected IRF-$1^{-/-}$ mice appeared moribund; this mouse had developed a large area of alopecia with multiple, firm, nodular skin

lesions, about 1 to 3 mm in diameter. No skin lesions were found on the other two IRF-1$^{-/-}$ mice, but they too were less alert and less active than the BCG-infected wild-type mice, which showed no clinical signs of disease. Histologically, the skin lesions of the one IRF-1$^{-/-}$ mouse were granulomas with a morphological pattern compatible with miliary cutaneous tuberculosis (*26*), which contained large numbers of acid-fast bacilli (Fig. 5A). Many acid-fast bacteria were also found in abundant granulomatous lesions in the livers (Fig. 5B), lungs, and spleens (*27*) of all three IRF-1$^{-/-}$ mice. In contrast, acid-fast rods were not found in the livers (Fig. 5C) and other organs of wild-type mice, and these organs contained smaller, and slightly fewer, granulomatous lesions than were found in IRF-1$^{-/-}$ mice (*27*). These findings indicate that, in comparison with wild-type mice, BCG infection is much more extensive and severe, and *M. bovis* elimination is impaired, in IRF-1$^{-/-}$ mice. Although it is tempting to conclude that the lack of NO generation by IRF-1$^{-/-}$ macrophages is responsible for the more severe course of BCG infection, other reasons cannot be ruled out. However, it seems unlikely that the more severe BCG infection was due to the reduced CD8$^+$ T cell numbers in the IRF-1$^{-/-}$ mice (*14*), because mice with a null mutation in the β_2-microglobulin gene that lack functional CD8$^+$ T cells are no different than control mice in their response to BCG infection (*28*).

This study identifies an essential role for IRF-1 in iNOS gene induction in macrophages. This role is specific for IRF-1, because macrophages from mice with a null mutation in the IRF-2 gene did release substantial amounts of NO_2^- on stimulation with IFN-γ and LPS (*27*). The demonstration that IRF-1 is required for iNOS gene activation explains the earlier postulated need for intermediary protein synthesis in iNOS mRNA induction by cytokines (*29*). The essential role for IRF-1 may make it possible to develop new strategies for enhancing or blocking NO generation through modulation of IRF-1.

REFERENCES AND NOTES

1. C. Nathan, *FASEB J.* **6**, 3051 (1992); C. J. Lowenstein and S. H. Snyder, *Cell* **70**, 705 (1992).
2. A. H. Ding, C. F. Nathan, D. J. Stuehr, *J. Immunol.* **141**, 2407 (1988).
3. C. F. Nathan and J. B. Hibbs Jr., *Curr. Opin. Immunol.* **3**, 65 (1991); F. Y. Liew and F. E. G. Cox, *Immunol. Today* **12**, A17 (1991); S. J. Green and C. A. Nacy, *Curr. Opin. Infect. Dis.* **6**, 384 (1993); K. P. Beckerman *et al.*, *J. Immunol.* **150**, 888 (1993).
4. K. D. Croen, *J. Clin. Invest.* **91**, 2446 (1993); G. Karupiah *et al.*, *Science* **261**, 1445 (1993).
5. Q.-W. Xie, R. Whisnant, C. Nathan, *J. Exp. Med.* **177**, 1779 (1993).
6. C. J. Lowenstein *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 9730 (1993).
7. T. Fujita, Y. Kimura, M. Miyamoto, E. L. Barsoumian, T. Taniguchi, *Nature* **337**, 270 (1989).
8. H. Harada *et al.*, *Cell* **58**, 729 (1989).
9. H. Harada *et al.*, *ibid.* **63**, 303 (1990).
10. L. F. L. Reis, H. Harada, J. D. Wolchok, T. Taniguchi, J. Vilček, *EMBO J.* **11**, 185 (1992).
11. N. Tanaka, T. Kawakami, T. Taniguchi, *Mol. Cell. Biol.* **13**, 4531 (1993).
12. R. Pine, T. Decker, D. Kessler, D. E. Levy, J. E. Darnell Jr., *ibid.* **10**, 2448 (1990).
13. T. Fujita *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9936 (1989); N. Watanabe, J. Sakakibara, A. Hovanessian, T. Taniguchi, T. Fujita, *Nucleic Acids Res.* **19**, 4421 (1991); A. Abdollahi, K. A. Lord, B. Hoffman-Liebermann, D. Liebermann, *Cell Growth Differ.* **2**, 401 (1991).
14. T. Matsuyama *et al.*, *Cell* **75**, 83 (1993).
15. D. E. Levy, D. S. Kessler, R. Pine, J. E. Darnell Jr., *Genes Dev.* **3**, 1362 (1989); T. C. Dale, A. M. A. Imam, I. M. Kerr, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1203 (1989); K. Shuai, C. Schindler, V. R. Prezioso, J. E. Darnell Jr., *Science* **258**, 1808 (1992).
16. R. Kamijo *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6626 (1993).
17. Q.-W. Xie *et al.*, *Science* **256**, 225 (1992).
18. M. A. Collart, D. Belin, J.-D. Vassali, S. De Kossodo, P. Vassali, *J. Exp. Med.* **164**, 2113 (1986); B. Beutler, V. Tkacenko, I. Milsark, N. Krochin, A. Cerami, *ibid.*, p. 1791.
19. Y. Vodovotz, C. Bogdan, J. Paik, Q.-W. Xie, C. Nathan, *ibid.* **178**, 605 (1993).
20. T. Fujita, H. Shibuya, H. Hotta, K. Yamanishi, T. Taniguchi, *Cell* **49**, 357 (1987).
21. D. J. Stuehr and M. A. Marletta, *J. Immunol.* **139**, 518 (1987); S. J. Green, M. S. Meltzer, J. B. Hibbs Jr., C. A. Nacy, *ibid.* **144**, 278 (1990).
22. S. J. Green *et al.*, *Infect. Immun.* **61**, 689 (1993).
23. R. V. Blanden, M. J. Lefford, G. B. Mackaness, *J. Exp. Med.* **129**, 1079 (1969); M. Pelletier, A. Forget, D. Bourassa, P. Gros, E. Skamene, *J. Immunol.* **129**, 2179 (1982).
24. V. Kindler, A.-P. Sappino, G. E. Grau, P.-F. Piguet, P. Vassalli, *Cell* **56**, 731 (1989); D. K. Dalton *et al.*, *Science* **259**, 1739 (1993); R. Kamijo *et al.*, *J. Exp. Med.* **178**, 1435 (1993).
25. J. Chan, Y. Xing, R. S. Magliozzo, B. R. Bloom, *J. Exp. Med.* **175**, 1111 (1992).
26. D. Dungworth, in *Pathology of Domestic Animals*, K. V. Jubb, P. C. Kennedy, N. Palmer, Eds. (Academic Press, San Diego, ed. 3, 1985), vol. 2, pp. 413–556.
27. R. Kamijo *et al.*, unpublished data.
28. J. L. Flynn, M. M. Goldstein, K. J. Triebold, B. Koller, B. R. Bloom, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12013 (1992).
29. W. Deng, B. Thiel, C. S. Tannenbaum, T. A. Hamilton, D. J. Stuehr, *J. Immunol.* **151**, 322 (1993).
30. Homozygous IRF-1$^{-/-}$ mice (*14*) and wild-type (129/SV/EV × C57BL/6 × DBA2) mice, aged between 2 and 12 months, were used in the experiments. (C57BL/6 × DBA2)F_1 mice (Jackson Laboratory, Bar Harbor, ME) were used as wild-type controls in some experiments. Mice were killed 3 to 4 days after intraperitoneal injection with 2 ml of 4% thioglycollate broth (Sigma). Peritoneal exudate cells, harvested in 10 ml of phosphate-buffered saline (PBS), were washed and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), penicillin (100 U/ml), and streptomycin (100 μg/ml) (complete medium). The endotoxin level of the FBS lot used was 0.3 endotoxin units per milliliter, as determined by the Limulus amebocyte lysate assay. Adherent monolayers were obtained by plating 1×10^5 cells per well in 96-well microplates. Peritoneal cells were adhered for 1 hour at 37°C in 5% CO_2 and washed three times with complete medium to remove nonadherent cells. Adherent monolayers were treated with cytokines, with or without LPS, for 48 hours. NO_2^- concentration in the medium was measured by a microplate assay method (*2, 16*).
31. P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987).
32. D. Caput *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1670 (1986).

33. We thank C. A. Nacy and C. Nathan for helpful advice; T. A. Kimmel and P. Schwenger for critical reading of the manuscript; T. Fujita, M. Tsujimoto, G. J. Thorbecke, Q.-W. Xie, C. Nathan, and B. Beutler for reagents and constructs; J. Hirst for flow cytometry; A. Dela Torre for photography; L. Richards for histological preparation; A. Feliciano and J. U. Aniagolu for technical assistance; and I. M. Totillo for preparation of the manuscript. Supported by a grant from the Human Frontier Science Program, grants R35CA49731 and P30CA13343 from the National Cancer Institute, grant AI28993 from the NIH, grants from Special Project Research Cancer Bioscience of Japan, Takeda Science Foundation, the Medical Research Council of Canada, and the National Science and Engineering Research Council of Canada. Animal care was in accord with institutional guidelines.

18 October 1993; accepted 11 January 1994

Positive and Negative Thymocyte Selection Induced by Different Concentrations of a Single Peptide

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T lymphocyte maturation is dependent on interactions between the T cell receptor (TCR) expressed on the developing thymocyte and intrathymic major histocompatibility complex (MHC)–peptide ligands. The relation between the peptide-MHC complex that results in negative or positive selection has not been identified. Here, the requirements for the maturation of thymocytes expressing a defined transgenic TCR specific for a viral peptide are studied in fetal thymic organ culture. Low concentrations of the viral peptide antigen recognized by this transgenic TCR can mediate positive selection, whereas high concentrations result in thymocyte tolerance. These findings support the affinity-avidity model of thymocyte selection.

Only 5% of the immature T cells generated in the thymus develop to form part of the peripheral T cell repertoire (*1*). These cells are selected by interactions between the TCR and MHC class I or class II molecules present on cortical thymic epithelial cells (*2, 3*). Positive selection depends on the presence of CD4 and CD8 coreceptors (*4*),